Na,K-ATPase: Comparison of the Cellular Localization of α-Subunit mRNA and Polypeptide in Mouse Cerebellum, Retina, and Kidney

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A clone encoding mouse brain Na,K-ATPase α-subunit was isolated from a mouse brain lambda gt11 cDNA library by using antisera to mouse and bovine brain α-subunit. A comparison of the nucleotide sequence of this clone with published sequences of rat brain α-subunit isoform clones showed it to be most similar to rat brain α1. An RNA antisense probe prepared from the cDNA insert of the mouse clone detected a single mRNA of approximately 4.5 kb in Northern blots of mouse brain and kidney RNAs. This probe hybridized only to an α1-cDNA insert from rat brain under high stringency conditions on Northern blots. The RNA antisense probe was used for in situ hybridization to sections of mouse kidney, cerebellum, and retina, and the cellular distribution of α-subunit mRNA (α-mRNA) was compared with that of α-subunit polypeptide (α-subunit) detected by immunofluorescence in similar sections. In kidney, α-mRNA distribution closely paralleled that of the polypeptide with abundant expression in ascending thick limbs and cortical distal tubules and weaker labeling in cortical proximal tubules. The co-distribution of α-mRNA and polypeptide in kidney where Na,K-ATPase localization is well established is consistent with the specificity of these probes. In the retina, prominent labeling with both probes was seen in photoreceptor inner segments, inner nuclear layer, and ganglion cell bodies. Plexiform layers and optic fibers expressed abundant α-subunit but little mRNA. Light labeling for both was seen in the outer nuclear layer. In cerebellum, α-mRNA and α-subunit were associated with soma of granule cells, basket cells, and stellate cells. Glomeruli and basket terminals contained abundant α-subunit but exhibited little reactivity with the riboprobe. In Purkinje cell bodies, in contrast, the antibody used to identify the cDNA clone did not resolve significant polypeptide in the somal plasmalemma despite abundant somal mRNA expression. Comparison of distribution of the two probes in cerebellum and retina indicates that message accumulation is primarily in cell bodies, while α-subunit epitopes may be co-expressed in cell bodies and/or transported to distant sites in cell-specific patterns.

Key words: sodium pump, in situ hybridization, immunocytochemistry

INTRODUCTION

Na,K-ATPase is the membrane-bound enzyme that exchanges intracellular Na^+ for extracellular K^+ in animal cells (Skou, 1988). The resulting Na^+ and K^+ gradients are critical for many general physiological processes, including regulation of cell volume and ion content. In addition, Na,K-ATPase activity is necessary for specialized functions unique to particular tissues, including maintenance of membrane electric potentials in excitable tissues and salt and water transport in osmoregulatory and exocrine epithelia. In accordance with its central role in such functions, high levels of the enzyme are present in tissues such as kidney and brain (Albers et al., 1989). Because of the different functions related to cation transport in these tissues and cell types, it is considered likely that there are cell-specific regulatory factors for this enzyme.

The enzyme consists of two polypeptide subunits, an α-subunit of about 110 kDa containing the catalytic site (Albers et al., 1989) and a β-subunit of about 40 kDa whose function may be to stabilize and provide mem-

brane insertion for the α-subunit (Noguchi et al., 1987; Kawakami and Nagano, 1988). Two α-subunit isoforms, α1 (previously termed α, lower Mr) and α2 (previously termed α +, higher Mr), with different electrophoretic mobility on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), have been resolved in the brain and several other tissues of various animal species (Sweadner, 1979; Matsuda et al., 1984; Morohashi and Kawamura, 1984; Lytton et al., 1985; Siegel et al., 1986; Stahl, 1986; Charlemagne et al., 1987).[1] Evidence from studies of genomic libraries suggest that four to five genes code for α-subunit isoforms in man (Shull and Lingrel, 1987; Sverdlov et al., 1987). cDNAs for three separate α-subunit isoforms, termed α1, α2, and α3, have been identified in rat brain (Shull et al., 1986; Herrera et al., 1987), and sequences coding for these isoforms have been mapped to three separate chromosomes in the mouse by using the rat isoform cDNAs as probes (Kent et al., 1987). Both α2- and α3-cell-free translation products share common epitopes and comigrate at the same rate on SDS-PAGE (Schneider et al., 1988) so that the slower-moving band as seen by protein or immune staining (termed α + or α2) may be composed of both isoforms.

A number of reports have presented data concerning differential tissue expression of isoform-specific mRNAs by using blot hybridization methods (Emanuel et al., 1987; Herrera et al., 1987; Kent et al., 1987; Young and Lingrel, 1987; Schneider et al., 1988). Although important information concerning expression at the tissue or organ level is obtainable from such studies, differential expression of isoform-specific mRNA at cellular levels requires high-resolution in situ hybridization techniques. At present there is only a single report of in situ hybridization of α-subunit isoform mRNA (Schneider et al., 1988). This survey examined expression of mRNAs of 18-day fetal rat tissues in whole body sagittal sections and also employed a probe for α3-mRNA to study regional expression in adult nervous system. α3-mRNA was localized in various portions of the forebrain and was largely restricted to the Purkinje cell layer in cerebellum (Schneider et al., 1988).

In order to study α-subunit gene expression in the nervous system, we examined in parallel the cellular distribution of α-subunit mRNA by high-resolution in situ hybridization and of α-subunit polypeptide by immunocytochemistry. Mouse cerebellum and retina were selected as examples of well-defined CNS structures, and kidney was used to further verify probe specificity. We found proportionate colocalization of an α1-mRNA probe with α polypeptide immunoreactivity in perikarya

of most cell types in cerebellum, retina, and kidney but not in neuronal extensions which were enriched in polypeptide. In Purkinje cells, however, abundant perikaryal mRNA was not accompanied by somal plasmalemma immunoreactivity, suggesting a different pattern of polypeptide expression or routing in these cells. A preliminary report of some of these data was published in abstract form (Hieber et al., 1988).

MATERIALS AND METHODS
Materials

Mice were purchased from Harlan Sprague Dawley, Inc. The mouse brain lambda gt11 library was from Clontech. Rat α-subunit isoform clones were generous gifts from Robert Levenson (Yale University, New Haven, CT) and Jerry Lingrel (University of Cincinnati College of Medicine, Cincinnati, OH). pGEM vectors and in vitro transcription kits were from Promega Biotec. Restriction enzymes and other enzymes used for subcloning and sequencing were from Bethesda Research Laboratories (BRL) or Boehringer Mannheim. Radioisotopes were purchased from Amersham. Prestained protein standards for electrophoresis were obtained from BRL and Sigma while SDS (catalogue #L-5750) was obtained from Sigma. Immunochemicals were obtained from ICN Biomedicals, Inc.

Preparation of Na,K-ATPase and Antisera Against α-Subunit

Mouse brain. The antimouse brain α-subunit antiserum (27C) was prepared and characterized as previously described (Siegel et al., 1984, 1986; Ernst et al., 1986).

Bovine brain. Sucrose-gradient fractions enriched in Na,K-ATPase were prepared from bovine brain and α-subunits were resolved on slab gels (160 × 140 × 1.5 mm) of 6% polyacrylamide as described (Siegel et al., 1986). For preparation of α-subunits for inoculation, 20% sucrose-gradient enzyme (Siegel et al., 1984, 1986), 3 mg protein, was applied to the whole slab for electrophoresis. Horizontal strips of gel containing combined α1 and α2 were sliced out of the unfixed, frozen gel and the polypeptides were electroeluted with an ISCO Electrophoretic Sample Concentrator model 1750. Rabbit polyclonal antibody (31B) was generated by methods described previously (Siegel et al., 1984) and specificity was established on immunoblots and by immunocytochemistry (see Results). SDS-PAGE (Laemmli, 1970), electroblotting (Towbin et al., 1979), and immunostaining procedures were performed as described (Siegel et al., 1986).

Isolation and Characterization of cDNA Clones

A mouse brain lambda gt11 expression library was plated with bacterial strain Y1090 at approximately

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[1] The faster-moving band on electrophoresis is referred to as α1 and the slower-moving band as α2 (see also Schneider et al., 1988).

5,000 plaque-forming units per 100 mm plate. The library was grown and the plaques screened with polyclonal antisera against mouse brain and bovine brain α-subunit according to the procedure of Young and Davis (1985).

Antisera were used for library screening at a 1:500 dilution. The bovine α-subunit antiserum was used for initial screening of the library because it was available in a larger quantity. Clones reactive with the bovine antisera were plaque-purified and tested with the mouse α-subunit antiserum, which was available in only a limited amount. Those reacting with both antisera, and therefore more likely to represent α-subunit clones, were used for further studies. Bacteriophage DNA was isolated from plate lysates (Maniatis et al., 1982), and cDNA inserts from the bacteriophage DNA were subcloned into M13 vectors mp18 and mp19. The inserts were isolated from the intracellular bacteriophage RF for probe synthesis and restriction enzyme analysis. The single-stranded extracellular bacteriophage was used for sequencing.

DNA Sequence Analysis

DNA sequence analysis was performed by the dideoxynucleotide chain-termination method of Sanger et al. (1977). Subclones of each cDNA insert in opposite orientation in the sequencing vectors were sequenced from the ends toward the center of the insert. Average lengths of 200–300 nucleotides were obtained. Each region was sequenced at least twice to confirm sequences. Sequences were combined and analyzed, translated, and compared with available ATPase sequences by using the Protein Identification Resource (Biomedical Research Foundation, Georgetown University Medical Center) (George et al., 1986).

Probe Synthesis

Several types of probes were prepared and used in these studies. Initially cDNA inserts isolated from mp18 and mp19 vectors were nick-translated (Weinstock et al., 1978) in the presence of ^{32}P-dCTP for Southern blot studies. For Northern blot and in situ hybridization studies, a cDNA insert of 483 bp, corresponding to the middle of the coding region for the rat α1-isoform, was inserted into the pGEM4 vector. RNA probes were prepared by transcription from the linearized vector in the presence of labeled nucleotide by using either T7 or SP6 polymerase to produce the sense or antisense transcripts as described previously (Bevilacqua et al., 1988); ^{32}P-labeled transcripts were prepared by polymerization in the presence of ^{32}P-UTP- and ^{35}S-labeled transcripts by polymerization in the presence of ^{35}S-thio-labeled nucleoside triphosphates.

Northern and Southern Blot Analysis

Total cellular RNA was isolated from brain and kidney of 3-week-old DBA mice by the method of Strohman et al. (1977). Total RNA was separated on 1% agarose gels containing 2.2 M formaldehyde according to the procedure of Lehrach et al. (1977). The RNA was transferred to GeneScreen membranes (New England Nuclear). Hybridization to labeled probes was carried out at 65°C with prehybridization, hybridization, and subsequent washing of filters as described previously (Bevilacqua et al., 1988).

Restriction fragments from rat α-isoform cDNAs were separated on 1% agarose gels in Tris-acetate-EDTA buffer (Maniatis et al., 1982) and subsequently transferred to GeneScreen membranes (New England Nuclear). Hybridization to labeled probes was carried out at 42°C in 50% formamide (Meinkoth and Wahl, 1984) for 18–24 hr. Low-stringency washing was done in 2 × SSC-0.1% SDS for 10 min at room temperature, in 1 × SSC-0.1% SDS for 10 min at 50°C, and finally in 0.5 × SSC-0.1% SDS for 1 hr at 50°C. High-stringency washes were subsequently carried out in 0.1 × SSC-0.1% SDS for 1 hr at 65°C. Hybridization was detected by autoradiography by using Kodak XAR-5 x-ray film.

Tissue Preparation

Mice (CBA strain) were anesthetized by intraperitoneal injection with sodium pentobarbital and perfused via the left ventricle for approximately 1 min with phosphate-buffered saline (PBS) and then with approximately 20 ml of 2–4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) over a 5 min period. Kidney, retina, and cerebellum were removed, cut into small tissue blocks, and postfixed in the same fixative for 2 hr. Fixed tissues were rinsed in buffer and then infiltrated with 20% sucrose in phosphate buffer and frozen in a mixture of acetone, hexane, and dry ice. Sections (10 μm) were cut in a cryostat and mounted on slides coated with either gelatin or poly-L-lysine.

In Situ Hybridization

The procedures employed for in situ hybridization followed those described by Watson et al. (1987). Briefly, cryostat sections of formaldehyde-fixed tissues were deproteinated with proteinase K (1 μg proteinase/ml of 100 mM Tris-HCl buffer, pH 8.0, containing 50 mM EDTA) for 30 min at 37°C and rinsed with distilled water. Sections were then incubated for 1 min in 0.1 M triethanolamine-HCl (pH 8.0) and for 10 min at room temperature in 0.1 M triethanolamine-HCl (pH 8.0) diluted with acetic anhydride (final concentration 0.25%, vol/vol) to reduce electrostatic binding of probes. After washing for 5 min in 2 × SSC, sections

were dehydrated through increasing concentrations of ethanol and air-dried. Sections were covered with an aliquot of hybridization buffer (final concentrations of 75% formamide, 10% dextran sulfate, 3 × SSC, 50 mM sodium phosphate buffer pH 7.4, 1 × Denhardt's solution, 0.1 mg/ml yeast tRNA, and 0.1 mg/ml sonicated, denatured salmon sperm DNA) containing approximately 1 × 10^6 cpm of the radioactive probe and sealed with coverslips. Dithiothreitol (10 mM) was added for ^{35}S-labeled probes. Sections were hybridized for 21 hr at 50°C. Coverslips were then removed, and the sections were washed in 2 × SSC for 5 min at room temperature. Sections were then incubated for 30 min at 37°C with RNase A, 200 μg/ml, in 0.5 M NaCl and 10 mM Tris-HCl buffer (pH 8.0). The sections were further washed in 2 × SSC for 10 min, in 1 × SSC for 10 min, in 0.5 × SSC for 60 min at 50°C, and finally in 0.5 × SSC for 10 min at room temperature.

Autoradiography

For rapid detection of hybridization with 32P-labeled probes, slides were air-dried and exposed in a cassette to Kodak XAR-5 x-ray film for 24–48 hr. High-resolution autoradiograms with ^{35}S-labeled probes were produced by dipping dried sections in Kodak NTB-2 emulsion (diluted 1:1 with distilled water), exposing the sections at 4°C until the desired grain density was obtained (3–6 days), and then developing the emulsion with D-19 developer. Sections were analyzed and photographed with darkfield, differential interference contrast (DIC), and phase-contrast optics.

Immunofluorescence Studies

Cryostat sections were exposed to 1% normal goat serum in phosphate-buffered saline (NGS/PBS) followed by incubation with bovine α-subunit antiserum 31B for 1 hr at room temperature and 12 hr at 4°C in a moist chamber. Serial dilutions of primary antibody with NGS/PBS were used to establish the optimal dilution for each antibody. Sections were rinsed with NGS/PBS and then exposed for 1 hr at room temperature to FITC-conjugated goat antirabbit IgG diluted 1:50 with NGS/PBS. Sections were washed with PBS, coverslipped with a mixture of 90% glycerol, 10% PBS, and 0.1% p-phenylenediamine, and examined and photographed with a Leitz fluorescence microscope.

RESULTS

Characterization of Bovine Brain α-Subunit Antiserum

Specificity of antiserum to bovine brain α-subunit (31B) was tested on immunoblots of mouse and bovine brain whole homogenates and fractions. Bands identified

as α1- and α2-isoforms were specifically stained in mouse and bovine brain whole homogenates (Fig. 1A, lanes 3,4) and in the enriched sucrose gradient fractions (Fig. 1B, lanes 3,4). None of the other proteins in the whole homogenates or fractions was significantly stained by the antiserum (compare to Coomassie blue stain for protein in Fig. 1, lanes 1,2).

Isolation and Analysis of Mouse Brain α-Subunit cDNA

Approximately 50,000 plaques from a lambda gt11 cDNA expression library constructed from brain mRNA of adult Swiss-Webster mice were screened, and eight clones were isolated and purified after being shown to react with antiserum 31B to bovine α-subunit. All clones reacting positively with 31B also reacted with antiserum to mouse brain (27C) α-subunit. The clones contained inserts of 100–650 bp in length. These inserts were subcloned directly into M13 sequencing vectors mp18 and mp19 and sequenced.

Because of the previously shown homology between α-isoforms of different species (Shull et al., 1985, 1986; Kawakami et al., 1986; Ovchinnikov et al., 1986; Herrera et al., 1987), the mouse brain cDNA sequences obtained in this study were compared with those published for rat brain α-isoforms (Shull et al., 1986; Herrera et al., 1987). Seven of the isolated clones showed similarity to rat brain α-isoforms. A clone of 483 bp (designated mb4a) showing sequence similarity to rat brain α1 was used to develop a probe for in situ hybridization studies. This clone was chosen for such use because of its length, being larger than all but one of the other positively reacting clones, and its similarity to rat α1, suggesting a mouse α-isoform which should be present in all tissues examined. By comparison with rat brain α1, mb4a extends from the middle of the H4 hydrophobic region through the phosphorylation site and ends 45 bp upstream from the FITC binding site. An alignment of a portion of the nucleotide sequence of mb4a extending from the 5' end of the cDNA through the phosphorylation site with that of the rat brain α1 sequence for this region is shown in Figure 2. This region was sequenced numerous times to resolve all ambiguities. A comparison of the total sequence of mb4A to published rat brain α-isoform sequences taking all ambiguities into account revealed >80% identity at the nucleotide level and 90% similarity at the amino acid level to rat brain α1, >78% identity at the nucleotide level and 88% similarity at the amino acid level to rat brain α2, and >75% identity at the nucleotide level and 85% similarity at the amino acid level to rat brain α3. The percent identity between the nucleotide sequences for the coding regions of the rat α isoforms themselves ranged from 76% to 79% (Shull et al., 1986). A comparison of the

A

B
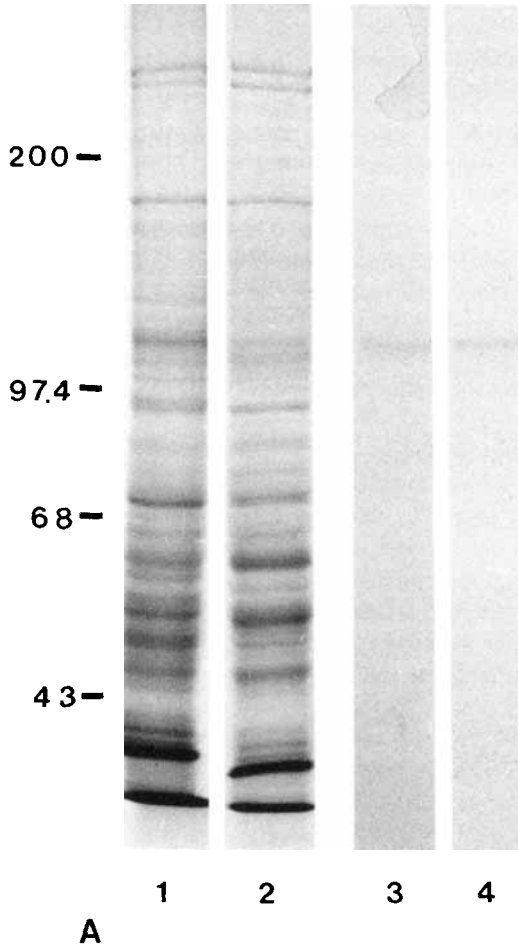
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Val Pro Glu Gly Leu Leu Ala Thr Val Thr
GTG CCG GAA GGT CTC CTA GCC ACC GTG ACG
--- --- --- -T- TTG --G --- --- --C ---
                Val


Val Cys Leu Thr Leu Thr Ala Lys Arg Met
GTT TGC CTG ACG CTC ACA GCC AAG CGC ATG
--A --T --- --- --- --T --- --- --- ---
_____


Ala Arg Lys Asn Cys Leu Val Lys Asn Leu
GCT CGT AAG AAC TGC CTG GTG AAG AAC CTG
--C A-G --- --- --- --C --- --- --- ---


Glu Ala Val Glu Thr Leu Gly Ser Thr Ser
GAA GCT GTG GAG ACT TTG GGG TCC ACG TCC
--- --- --- --- --C --- --- --- --A ---

                              P
Thr Ile Cys Ser Asp Lys Thr Gly 379
ACC ATC TGC TCT GAC AAA ACT GGA
--- --- --- --C --G --G --- ---
```

2

Fig. 2. Nucleotide sequence analysis of mb4a cDNA. The nucleotide and deduced amino acid sequence of a portion of the clone are compared with those of the corresponding region of rat brain α1 subunit. Differences from the rat nucleotide and amino acid sequences are indicated below the sequences for the mouse clone. The amino acid sequence starts with amino acid 332 in the middle of H4 hydrophobic region of the rat sequence and extends past the phosphorylation site to amino acid 379. The H4 hydrophobic region is underlined and the phosphorylation site is indicated by the letter P.

mb4a sequence to that of rat stomach H,K-ATPase (Shull and Lingrel, 1986) indicated much less homology, with 65% identity at the nucleotide level and 75% at the amino acid level.

Comparison of Mouse With Rat α-Isoform cDNAs

The similarity of the mouse clone to the rat α1-isoform of Na,K-ATPase was also tested by hybridization of the mouse cDNA probe under stringent conditions to cDNA inserts coding for the three rat brain α-isoforms (Fig. 3A). Under these conditions, the probe hybridized to the rat α1-isoform only. Under the lower-stringency post-hybridization washing conditions (Fig. 3B), reaction with α2- and α3-cDNA inserts was also seen but was much less intense than with α1. This probe hybridized strongly with restriction fragments from the middle

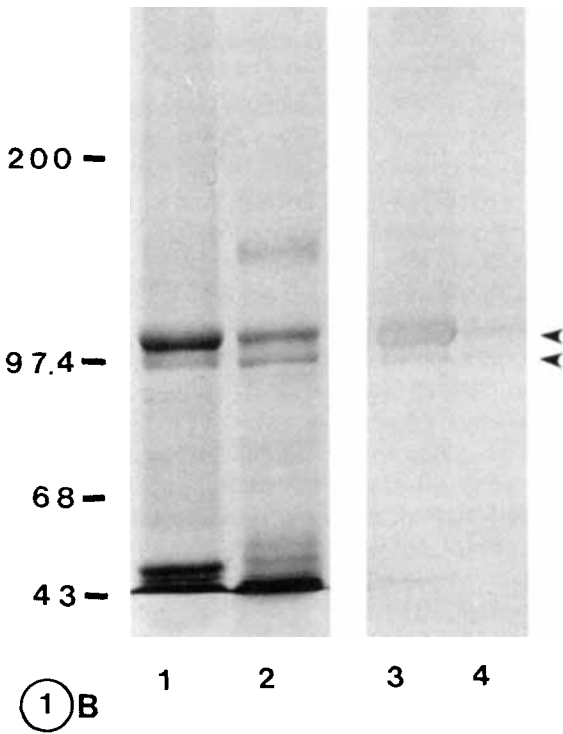Fig. 1. Immunoblots with antibovine brain Na,K-ATPase catalytic (α1 + α2) subunit. **A:** Whole brain homogenates. **B:** 20% sucrose gradient brain fractions enriched in Na,K-ATPase. **Lanes 1 and 2:** Coomassie blue stain. **Lanes 3 and 4:** Immunoblot with 31B antiserum, 1:500 dilution. Mouse brain, lanes 1 and 3; bovine brain, lanes 2 and 4. Arrowheads indicate α2 (higher Mr) and α1 (lower Mr). Little or no α1 is seen in the whole homogenates (A) because of its relatively low concentration, but substantial amounts of α1 together with α2 are seen in the enriched fractions, and the ratio of α2:α1 is higher in mouse than in bovine brain (B).
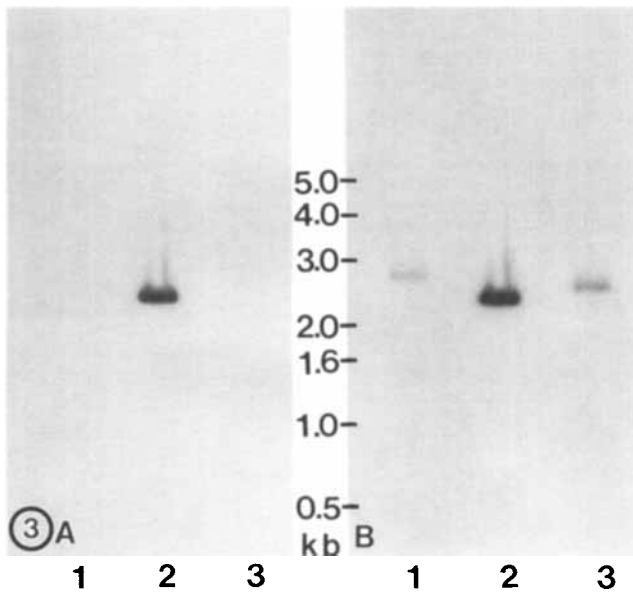
Fig. 3. Hybridization of mouse cDNA probe to restriction fragments of rat α-isoform clones. **A:** Stringent conditions. **B:** Nonstringent conditions. **Lane 1:** Rat α3. **Lane 2:** Rat α1. **Lane 3:** Rat α2. Molecular weight standards in kb are indicated in the middle.

of the coding region of the rat α1-cDNA containing the H4 hydrophobic region and phosphorylation site and weakly with restriction fragments from rat α2- and α3-cDNAs containing these regions. No reaction was found with other restriction fragments outside this region (data not shown).

Northern Blot and In Situ Hybridization Studies With ^{32}P-Riboprobes

The cDNA insert from clone mb4a was transferred to the vector pGEM 4 and ^{32}P-labeled sense and antisense RNA transcripts were prepared. Hybridization of the RNA probes to Northern blots revealed a single band of approximately 4.5 kb in brain and kidney RNAs with the antisense probe and no hybridizing bands with the sense probe (Fig. 4). Bands of identical size were detected on a similar Northern blot by using rat α1-cDNA as a probe (data not shown). The same ^{32}P-labeled probe was used for in situ hybridization with sections of mouse kidney and retina. A strong hybridization signal was resolved in both tissues with the antisense probe (Fig. 5A). Resolution in kidney was sufficient to indicate a regional distribution of probe similar to that seen previously for catalytic subunit by cytochemistry (Ernst and Schreiber, 1981) and immunocytochemistry (Siegel et al., 1984; Kashgarian et al., 1985)—namely, intense labeling of outer medulla, moderate reactivity of cortex, and undetectable labeling of inner medulla. No hybridization

was observed in sections exposed to the sense probe (Fig. 5B).

In Situ Localizations With ^{35}S-Riboprobe and α-Subunit Antiserum

A ^{35}S-labeled antisense probe was prepared for in situ hybridization to sections of mouse kidney, cerebellum, and retina to permit detection of regions of hybridization with greater resolution. Probe distribution in autoradiograms of the tissues was viewed with darkfield, DIC, and phase optics and was compared to the distribution of α-subunit as revealed by immunofluorescent staining with 31B. There was no specific immunofluorescence staining with preimmune serum controls (data not shown).

Kidney. In kidney, both the mRNA (Fig. 6A) and the polypeptide (Fig. 6B) probes strongly labeled tubules in the outer medulla, whereas tubules in the inner medulla were not labeled above background levels. With DIC optics, the regional distribution of riboprobe was resolved as intense labeling of ascending thick limbs in outer medulla (not shown) and their extensions as distal tubular segments in the cortex (Fig. 6C). In contrast, proximal tubules in the cortex were moderately labeled and collecting tubules were lightly labeled. At the cellular level, α-subunit mRNA appeared uniformly distributed in the cytoplasm. The tubule-specific pattern and intensity of mRNA distribution paralleled that of the α-subunit as revealed by immunofluorescence (Fig. 6B).

Retina. Distribution of α-mRNA and α-subunit among the various retinal layers is compared in the darkfield and fluorescence images shown in Figure 7A and 7B. More detailed resolution of probe distribution is seen in Figure 7C. Prominent labeling of mRNA with the antisense probe was detected in inner segments of the photoreceptor layer, in cell bodies of the inner nuclear layer, and in ganglion cell bodies of the ganglion cell/optic fiber layer (Fig. 7A,C). In these layers, distribution of mRNA was paralleled by intense α-subunit immunofluorescence (Fig. 7B). The outer nuclear layer of the photoreceptor cells showed scattered foci of mRNA label (Fig. 7A,C) and a reticular pattern of weak to moderate immunofluorescence which is probably associated with Müller cells (McGrail and Sweadner, 1986) and possibly with photoreceptor plasma membrane (Fig. 7B). The regions with high concentrations of cell processes and synapses composing the inner and outer plexiform layers were relatively free of α-subunit message (Fig. 7A,C). In marked contrast, the α-polypeptide was intensely expressed in both of the plexiform layers and in the optic nerve fibers (Fig. 7B).

Cerebellum. In cerebellar cortex, pronounced expression of α-subunit mRNA was visualized as focal accumulations of autoradiographic grains in molecular,

Purkinje cell, and granular layers, whereas white matter was unlabeled (Fig. 8A). As shown in phase contrast (Fig. 8B), Purkinje cell bodies were heavily labeled, as were basket cell and outer stellate cell bodies in the overlying molecular layer. There were areas of less intense labeling throughout the neuropil of the molecular layer, but resolution was not sufficient to discriminate between neuronal extensions and glial components. In the granular layer, autoradiographic grains were associated with granule cell perikarya, whereas glomeruli, which consist of multiple granule cell dendrites, a mossy fiber terminal, a Golgi cell axon, and astrocytic processes, were poorly labeled (Fig. 8B). Immunofluorescence localization of α-subunit polypeptide in mouse cerebellum with 31B (Fig. 8C) showed intense immunostaining of basket cell terminal regions associated with Purkinje cells. In contrast, immunofluorescence localization with 31B revealed little if any reactivity in Purkinje cell perikarya, despite the presence of abundant message (compare to Fig. 8A and 8B), or in proximal dendritic plasmalemma. In the granular layer, glomeruli and borders of granule cell perikarya were strongly immunoreactive for α-subunit polypeptide (Fig. 8C). Cell bodies in the molecular layer were also outlined by immunofluorescence and the surrounding neuropil was diffusely stained.

DISCUSSION

We have isolated a cDNA clone corresponding to a portion of mouse brain Na,K-ATPase α-subunit mRNA and have demonstrated its use for in situ studies of sodium pump expression at the transcriptional level in mouse brain, kidney, and retina. An antibody probe to the α-subunit was used in parallel studies to determine the distribution of the translated polypeptide.

Characterization of Probe

A Na,K-ATPase α1-subunit probe was chosen for these initial studies because of its widespread occurrence in animal tissues (Herrera et al., 1987; Kent et al., 1987; Young and Lingrel, 1987). This cDNA was selected from a lamda gt11 expression library because the fusion protein expressed by phage clones containing the cDNA reacted with polyclonal antisera to both bovine and mouse brain α-subunit. We conclude that the cDNA we have isolated corresponds to the α1-isoform based on the following lines of evidence: 1) sequence identity was greatest to rat α1-isoform in comparison to α2 and α3 (Shull et al., 1986; Herrera et al., 1987); 2) hybridization was strongest to restriction fragments from a rat α1-isoform clone as compared to similar fragments from α2 and α3 isoform clones (Fig. 3); 3) the cDNA clone hybridized to an abundant mRNA of approximately 4.5 kb in both mouse brain and kidney (Fig. 4). Rat α1-cDNA

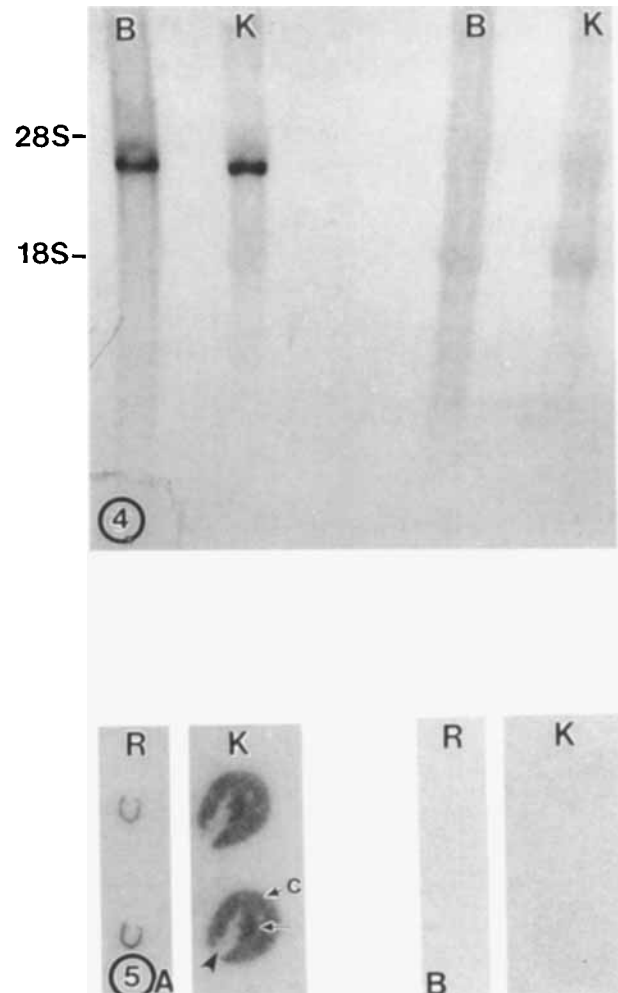Fig. 4. Northern blot analysis of mouse brain and kidney RNA with mouse α-subunit antisense and sense RNA probes. Total cellular RNA (20 μg) from mouse brain (B) and kidney (K) was electrophoresed in a 1% agarose/2.2M formaldehyde gel and transferred to GeneScreen. RNA homologous to the α-subunit was located by hybridization of RNA on the membrane to ^{32}P-labeled antisense (pair of lanes on left) and sense (pair of lanes on right) RNA probes (1 × 10^8 cpm/μg) followed by autoradiography. Positions of 28S and 18S ribosomal RNA markers are as indicated.

Fig. 5. In situ hybridization of ^{32}P-labeled mouse α-subunit RNA probes to mouse retina and kidney sections. Cryostat sections of mouse retina and kidney were prepared and hybridized to the antisense (A) and sense (B) RNA probes described in the legend to Figure 4. Sections were exposed directly to x-ray film for detection of hybridization. C, cortex; arrow, outer medulla; arrowhead, inner medulla.

has also been reported to hybridize to mRNA of this size in rat brain and kidney (Herrera et al., 1987; Kent et al., 1987). Since levels of mRNA for α2 and α3 are very low
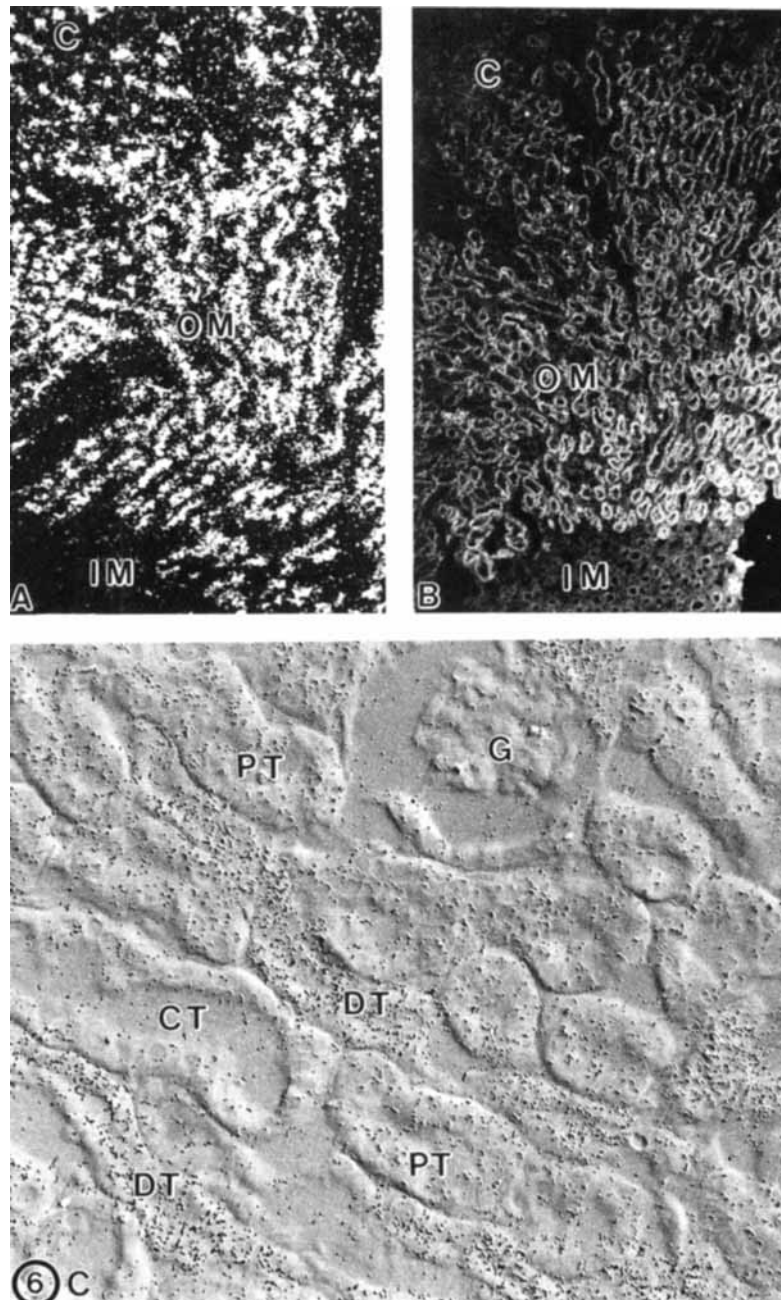
Fig. 6. Localization of α-subunit mRNA and polypeptide in mouse kidney. **A:** [35]S-labeled mRNA probe hybridized strongly with tubules in outer medulla (OM), identified histologically as thick ascending limbs, and scattered tubules in cortex (C). Tubules in the inner medulla (IM) were unlabeled. **B:** The distribution of immunostaining with 31B antiserum to the α-subunit paralleled that seen with the riboprobe. **C:** In thecortex, distal tubules (DT) expressed abundant α-mRNA, whereas proximal tubules (PT) and collecting tubules (CT) expressed moderate and low hybridization signals, respectively. G, glomerulus. A: autoradiogram, darkfield optics. ×70. B: epifluorescence. ×65. C: autoradiogram, DIC optics. ×345.

or nonexistent in adult rat kidney (Herrera et al., 1987; Kent et al., 1987), it is evident that the abundant target recognized by the probe in kidney is α1-mRNA.

Light reactivity of the mouse cDNA clone to rat α2 and α3 was seen only with low-stringency washing conditions following blot hybridization (Fig. 3B). This is to be expected since there is some sequence similarity to the rat α2- and α3-isoforms. Therefore, under the conditions of in situ hybridization using the mouse cRNA probe in this study (i.e., moderate stringency), some small
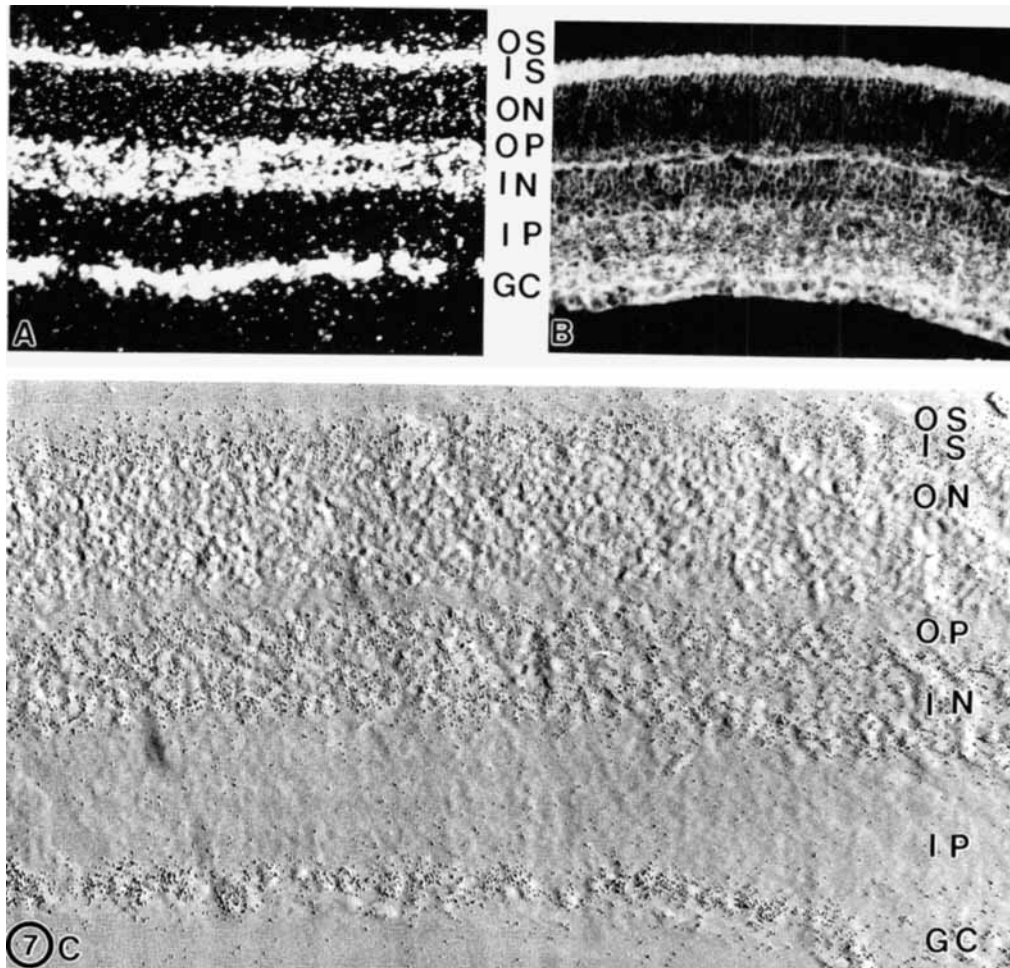
Fig. 7. Localization of α-subunit mRNA and polypeptide in mouse retina. In situ hybridization of [35]S-labeled α-subunit mRNA probe as revealed by darkfield (A) and DIC (C) optics is compared to immunofluorescence staining with 31B antiserum to α-subunit polypeptide (B). See text for description. OS, outer segments; IS, inner segments; ON, outer nuclear layer; OP, outer plexiform layer; IN, inner nuclear layer; IP, inner plexiform layer; GC, ganglion cell layer. A, ×165. B, ×150. C, ×330.

amount of detection of α2- and α3- mRNAs in addition to α1 cannot be excluded.

Localization in Kidney

The pattern of riboprobe distribution in kidney (Figs. 5, 6A,C) paralleled immunofluorescence localization with 31B (Fig. 6B) and previously reported immunoperoxidase staining with antiserum to mouse brain α-subunit (Siegel et al., 1984). The tubule-specific pattern and intensity of reactivity with both probes correspond to the distribution of Na,K-ATPase protein as seen by immunoelectron microscopy (Kashgarian et al., 1985) and enzymatic activity as revealed by histochemical methods (Ernst, 1975; Ernst and Schreiber, 1981). Taken together, the data strongly support the specificity of antiserum 31B and the riboprobe for sequences of the α-subunit protein

and message. It is presumed that all the mRNA label and immunoreactivity shown in the kidney is α1-isoform-specific, since all other known Na,K-ATPase mRNAs have been shown by blot hybridization to be absent or in very low quantity relative to α1 in adult rat kidney (Herrera et al., 1987; Young and Lingrel, 1987; Emanuel et al., 1987; Schneider et al., 1988). The relative abundance of mRNA was proportionate to that of polypeptide in each region of kidney. This suggests a coordination or linkage between mRNA synthesis and translation in this tissue. Within the various tubular cells, mRNA appeared uniformly distributed throughout the cytoplasm. The polarized distribution of protein to the basolateral membrane (Ernst, 1975; Ernst and Schreiber, 1981; Siegel et al., 1984; Kashgarian et al., 1985) was not reflected by a similar asymmetrical compartmentation of mRNA. Thus,
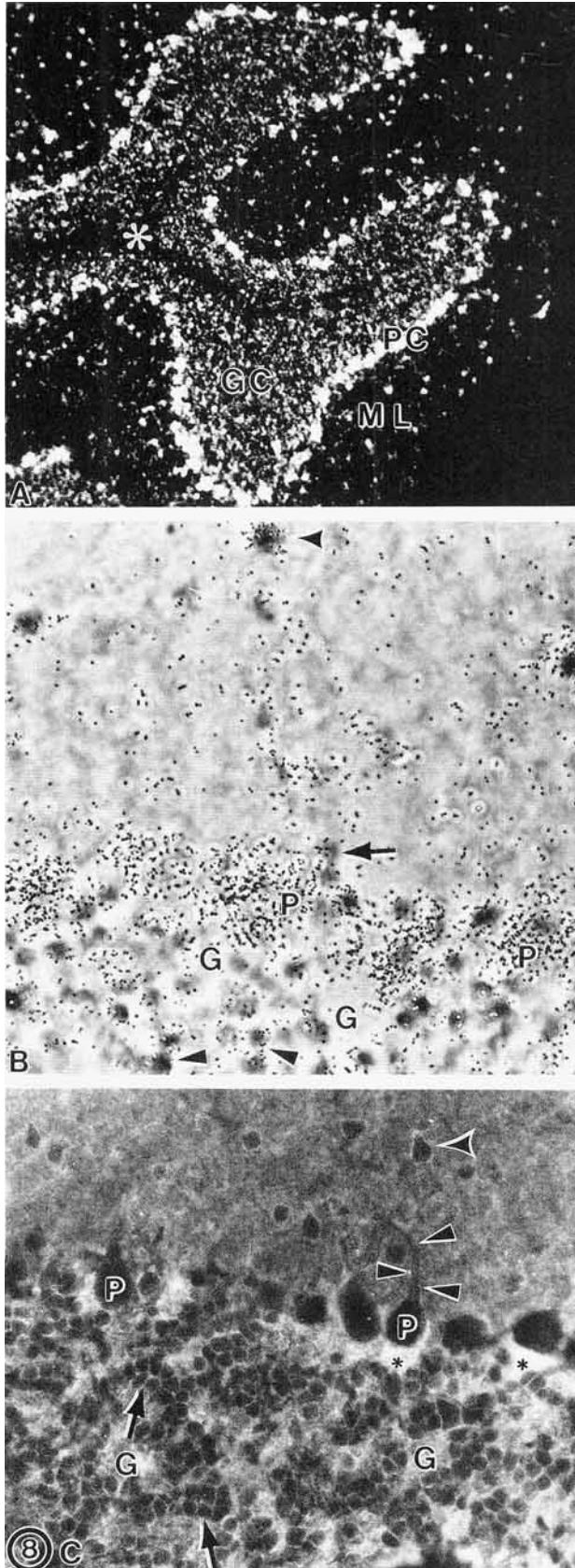
the routing mechanism for polarization of the membrane-bound enzyme begins with or after polypeptide synthesis.

Localization in Retina

In retina, α-mRNA and α-polypeptide are strongly expressed in the inner segments of photoreceptor cells (Fig. 7), which is the site responsible for the bulk of protein synthesis, and for Na,K-ATPase-dependent maintenance of the dark current (Barnstable, 1987). The inner nuclear layer and ganglion cells in the optic fiber layer also expressed abundant message (Fig. 7A,C) and, in agreement with the results of McGrail and Sweadner (1986), cell bodies and processes in these layers were strongly immunoreactive for the α-subunit (Fig. 7B). In addition, in studies of dissociated retinal cells with the antiserum used in this study, abundant α-subunit was resolved in ganglion cell and bipolar cell dendritic processes as well as in soma and axonal processes (unpublished data; see also McGrail and Sweadner, 1986). The virtual absence of resolvable α-mRNA in both plexiform layers (Fig. 7A,C) despite their abundant expression of α-subunit (Fig. 7B) is consistent with transport of Na,K-ATPase from sites of synthesis in neuronal cell bodies to destinations in cell processes and synapses (see Specht and Sweadner, 1984). It cannot be ruled out, however, that the amount of dendritic α-mRNA is below the resolution of light level autoradiography or that the isoform-specific mRNA in these dendrites differs from that expressed in perikarya.

Localization in Cerebellum

In cerebellar cortex, granule cells expressed α-mRNA (Fig. 8B) as well as polypeptide, as judged by immunofluorescence (Fig. 8C) and immunoperoxidase staining (Siegel et al., 1984). Because astrocytic velate processes envelop granule cell bodies and since the so-

Fig. 8. Localization of α-subunit mRNA and polypeptide in mouse cerebellum. **A:** The Purkinje cell layer (PC) exhibited a strong hybridization signal with the [35]S-riboprobe. Focal accumulations of autoradiographic grains were present in the molecular layer (ML) and the granular layer (GC). White matter (asterisk) was unlabeled. **B:** Intense labeling with the [35]S-riboprobe was seen in perikarya of Purkinje cells (P), basket cells (arrow), stellate cells (curved arrowhead), and granule cells (small arrowheads). Glomeruli (G) were poorly labeled. **C:** Immunostaining of α-subunit with 31B antiserum was pronounced in basket regions (asterisks) and glomeruli (G) and outlined granule cell (arrows) and basket/stellate cell (curved arrowhead) soma. Purkinje cell bodies (P) and their proximal dendrites (straight arrowheads) appeared unreactive. The neuropil of the overlying molecular layer was diffusely reactive. A: autoradiogram, darkfield. ×75. B: autoradiogram, phase contrast. ×625. C: epifluorescence. ×375.

mal cytoplasm in these cells is restricted to a thin peri-nuclear ring, some of the α-mRNA and α-polypeptide might be associated with astrocytic elements. Immuno-peroxidase studies at the electron microscope level with 31B have resolved α-subunit at the granule cell somal plasma membrane and in axonal and astrocytic velate processes in the granular layer (unpublished observations).

Basket cells also expressed α-mRNA (Fig. 8B), and basket regions composed of basket cell axonal terminals juxtaposed to the axon hillocks and initial axon segments of Purkinje cells were intensely immunoreactive for α-subunit (Fig. 8C; Siegel et al., 1984). A recent report (Schneider et al., 1988) described α3-mRNA cerebellar labeling in adult rats as largely restricted to the Purkinje cell layer. Inspection of Figure 3C in that paper suggests to us that labeling above background with their probe was also present in granular and molecular layers although Purkinje cell labeling was dominant. The current report clearly resolved labeled mRNA in adult mouse cerebellum within stellate, basket, and granule cells as well as abundant signal within Purkinje cell bodies (Fig. 8A,B). Because of the large size of Purkinje cells, their mRNA content appears particularly striking. However, the considerably smaller cytosolic volume of basket, stellate, and, particularly, granule cells, suggests that the distinct hybridization signal resolved in these cells may represent a relatively high degree of α-mRNA expression. Since the probe used in this study showed a large reaction with rat α1-cDNA and very little reaction with α2- and α3-cDNAs on blots, even at low stringency (Fig. 4B), the bulk of labeling in situ is attributed to α1-mRNA. These observations, taken together with the data reported for adult rat cerebellum by Schneider et al. (1988), suggest that multiple α-subunit isoform mRNAs (at least, α1 and α3) are expressed by some individual CNS cells. If so, the different isoforms might be translated or processed at different times, routed to different locations within the cell, or even combined in hybrid molecular assemblies.

As indicated above, Purkinje cell bodies showed abundant accumulation of α-mRNA (Fig. 8A,B). However, when the same antiserum (31B) that selected the cDNA clone was used, there was no clearly resolvable immunofluorescence localization of α-subunit polypeptide to Purkinje cell soma or to proximal portions of dendrites that emerge from these cell bodies (Fig. 8C). The staining that outlines Purkinje cell bodies seen in Figure 8C and in an earlier immunoperoxidase study (Siegel et al., 1984) appeared on close examination to be primarily related to juxtaposed basket terminals and glial enwrapments, and this interpretation is consistent with immunoelectron microscope studies with this antiserum (unpublished observations). In contrast, granule cells

and retinal photoreceptor inner segments and ganglion cells (see above) expressed both α-mRNA (Figs. 8B, 7C) and somal plasmalemma polypeptide (Figs. 8C, 7B). It is possible that the bulk of synthesized α-subunit in Purkinje cells is transported to and inserted in membranes of distal processes. If Na,K-ATPase is present in Purkinje somal plasmalemma, it is a different isoform than that detected in granule and retinal ganglion cell perikarya and photoreceptor inner segments.

SUMMARY

The data from kidney, retinal, and cerebellar localizations suggest close coupling between α1-mRNA accumulation and expression of catalytic subunit protein. In addition, the retinal and cerebellar studies suggest that in some cells, the bulk of polypeptide may accumulate at membranes that are remote from the primary site of translation. Whether isoform-specific differences in the routing to specific membrane domains also exist remains to be determined.

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